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IMMUNITY TO MONOMORPHIC TRYPANOSOMA BRUCEI: HUMORAL RESPONSE,(U)
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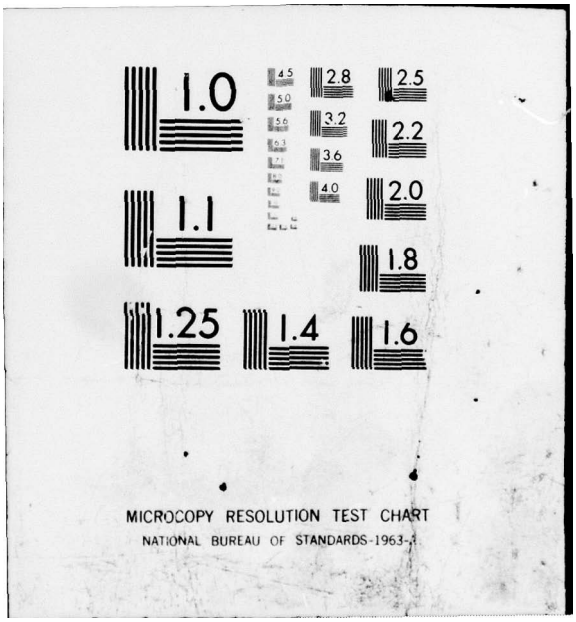
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(6) IMMUNITY TO MONOMORPHIC TRYPANOSOMA BRUCEI: HUMORAL RESPONSE*

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ABSTRACT

Trypanosome agglutinating antibodies, predominantly of the IgG class, are formed in T. brucei infected rats cured with Berenil (diminazene). The duration of Berenil prophylaxis following administration of a minimum curative dose is approximately 28 days. Rechallenge of drug-cured animals with homologous organisms results in the production of IgG, indicating an apparent absence of interference under these conditions with B-cell, T-cell cooperativity.

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Elevation in immunoglobulin(s), particularly of the IgM class, has been noted in the trypanosome infected host (Klein et al, 1970). The subsequent lack of a significant IgG response has been attributed to the frequency of antigenic variation, events which reinitiate the first sequence of the hosts primary antigenic response (Seed et al, 1969). An alternate view holds that trypanosome parasitemia elicits synthesis of non-specific IgM, i.e. antibodies lacking in specificity for the antigenic variants are made. IgM antibodies from trypanosome infected hosts have been shown to react or cross-react with heterologous antigens (Houba et al, 1969).

Recognition of the regulatory effects of T-cells on B-cells has led to renewed interest in the phenomenon of elevated IgM levels in trypanosome parasitemia. A breakdown in the regulatory effects of T-cells on B-cells during parasitemia would be consistent with the observed high levels of IgM and with the lack of long term immunological memory to reinfection. Impairment of general immunological recognition in the trypanosome parasitized host has been tested by noting the class of antibodies formed against heterologous antigens (Longstaffe et al, 1973; Murray et al, 1973; Terry et al, 1973).

In immunity induced by chemotherapeutic cure it is unclear whether a transient humoral response contributes to the post-cure refractory period (Cantrell, 1955). The relationship of post-cure protection to the class(es) of antibody that may be synthesized during or after this period has not been examined. Prophylaxis resulting from the use of Berenil (diminazene), an early acting diamidine class

drug, may be a consequence of its trace activity in blood (Cunningham and VanHoeve, 1964) and dosage used (Lumsden, Herbert and Hardy, 1965). Although protections of varying period have been described with different trypanocidal agents (Whiteside, 1962) effective post-cure immunity is generally of short duration (Desowitz, 1970). The method of immunizing animals by chemotherapeutic cure is subject to the criticism that protection may result from the presence of residual drug (or its proximate form) in the challenged host.

The experiments reported here characterize the humoral response of inbred rats to a monomorphic strain of T. brucei during infection, during chemotherapeutic cure, after cure and after rechallenge following drug-induced immunity. The contribution of Berenil prophylaxis during the refractory period was examined as was the presence or absence of trypanosome agglutinating antibodies occurring during infection, cure, post-cure and after rechallenge. The class of specific antibodies formed (IgM or IgG) was also examined.

MATERIALS AND METHODS

(i) Animals: 200g Fisher Strain 344 inbred male rats and NLW outbred 20g male mice were obtained from National Animal Laboratory Co., Creve Coeur, Mo. A monomorphic laboratory strain of Trypanosoma brucei was originally obtained from the East African Trypanosomiasis Research Organization (EATRO 691A). In laboratory rodents this strain gives a fulminating parasitemia resulting in death of the animal within 4 days and does not exhibit antigenic variation.

(ii) Buffers: TRIS-glucose EDTA buffer contained the following: 5g TRIS-base, 2g glucose, 4g NaCl, 0.2g KCl, 5g Na_2EDTA, and H_2O to 1 liter. The pH was adjusted to 7.4 with 0.1N HCl. TRIS-glucose buffer contained the following: 5g TRIS-base, 2g glucose, 4g NaCl, 0.2g $MgCl_2$, 0.2g $CaCl_2$ and H_2O to 1 liter. The pH was adjusted to 7.4 with 0.1N HCl. Phosphate buffered saline contained: 55.2g Ha_2HPO_4, 13.6g KH_2PO_4, and 22g NaCl in a final volume of 1 liter. The pH was adjusted to 7.8.

(iii) Routine Passage: Parasitemias were maintained by passage in 20-30g NLW male mice. Mice were infected with ~2×10^6 trypanosomes (in a volume of 0.2ml TRIS-glucose EDTA, pH 7.4) by intraperitoneal (i.p.) injection. Haemocytometry of tail blood samples was done two days after injection. When counts showed ~3×10^8 trypanosomes/ml blood cardiac puncture was performed utilizing heparin as anticoagulant. The blood was adjusted to a concentration of 10×10^6 trypanosomes/ml and passage was made by injection of 0.2ml into naive mice.

This procedure was repeated every two days.

(iv) Harvest of Blood Trypanosomes: Cardiac puncture was performed and the blood centrifuged at 600×g for 10 minutes at 4°C in a swinging bucket rotor (Sorvall, HB-4). The upper plasma layer was removed and a small quantity of TRIS-glucose buffer added so as not to disrupt the trypanosome layer. The trypanosomes were resuspended in the buffer by gentle agitation, removed, washed 2X in cold TRIS-glucose buffer by centrifugation, and finally resuspended in buffer. The washed trypanosomes were separated from contaminating blood cells by passage through a DEAE cellulose column eluted with TRIS-glucose buffer (Lanham, 1968). The final trypanosome suspension was counted by haemocytometry and the parasite concentration adjusted to the desired number/ml. Trypanosomes so purified were used to infect NLF rats. NLF rats to be used as a source of trypanosomes for agglutination titer tests were infected with 3×10^6 trypanosomes on day 0. On day 3 when the parasitemia showed $\sim10^9$ organisms/ml blood, cardiac puncture was performed and the trypanosomes purified as noted above, except that borate buffered saline (pH 7.8) was used as the final suspension medium for trypanosomes used in the agglutination tests.

(v) Quantitation: Parasitemia levels in infected animals were determined with a Brite Line haemocytometer and red cell diluting pipette. Tail blood was drawn to the appropriate level in the pipette and TRIS-glucose EDTA buffer (pH 7.4) was used as the diluting fluid. Cell counts were made at 430 magnifications. Purified trypanosomes

suspended in buffer were similarly quantified.

(vi) Drug Solutions. Antipyrene free Berenil (4,4'-Diamidinodiazobenzene diaceturate) was obtained from Farbwerke Hoechst Laboratory, Frankfurt. Drug solutions were always freshly made up in TRIS-glucose buffer, pH 7.4. Rats to be given a minimum curative dose (mcd) of drug were weighed to the nearest gram. A mcd was calculated based on 5mg/kg body weight. All injections were via the intraperitoneal (ip) route.

(vii) Determination of the Duration of Berenil Prophylaxis. On day 0 NLF rats were injected ip. with a mcd. On day 1, two rats that had received Berenil 24 hours earlier were challenged with an ip. injection of 100,000 trypanosomes contained in 0.2ml TRIS-glucose EDTA buffer. Control rats (no Berenil) were identically challenged. All animals were monitored daily for infection by means of blood smears until death ensued, or for 30 days after challenge if no infection developed. On day 2 the same procedure was repeated with a second pair of rats that had received drug 48 hours earlier. Controls were routinely challenged in an identical way. This procedure was carried out through day 40 after Berenil administration.

(viii) Preparation of Sera.

(a) Normal Control Sera. NLF rats were bled by cardiac puncture using a 10cc syringe fitted with a 20 gauge needle. The pooled blood was poured into 15ml centrifuge tubes and allowed to clot for one half hour at room temperature. The clots were rung with a sterile syringe needle and the blood samples refrigerated for

2 hours at 14°C. The sera were separated by centrifugation at 2,000 g in a HB-4 swinging bucket rotor, pipetted into 1 ml. vials, and stored at -20°C.

(b) Post-Berenil Control Sera. Uninfected NLF rats were given a mcd on day 0. On day one, three rats were bled and the sera harvested and stored as in (a). This procedure was repeated for days 3,5,7,9,11,13,15, and 17 after drug administration.

(c) Sera from Parasitized Animals. On day 0 rats were injected ip. with 50,000 trypanosomes. The rise in parasitemia was followed by blood smears and by haemocytometry. 24 hr. after infection 4ml blood samples were obtained from three rats by cardiac puncture. The blood was processed and the sera stored as in (a). This procedure was repeated on days 2,3, and 4 after infection with different groups of animals.

(d) Preparation of Sera from Rats Undergoing Treatment and after Berenil Cure. NLF rats were infected with 50,000 trypanosomes on day 0. Haemocytometry was performed on day 4. Rats were given a mcd on day 4. On day 5 three cured rats were bled by cardiac puncture and the blood was processed and the sera stored as in (a). This procedure was repeated on different groups of cured rats on days 6 through 23 after initial infection (days 2 through 19 after cure).

(e) Preparation of Sera from Rats Challenged to Determine Post-cure Immunity. NLF rats were infected on day 0, cured on day 4 and challenged with 100,000 trypanosomes on day 60. On day 2 after challenge blood smears were made. Groups of rats exhibiting no

parasitemia were bled by cardiac puncture on days 2,4,7,9,15, and 20 after rechallenge. The blood was processed and the sera stored as in (a).

(ix) Determination of Trypanosome Agglutinating Antibody Titer. An in vitro micro agglutination assay was used to determine specific anti-trypanosomal agglutinating antibodies. Sera samples prepared in (viii) were decomplemented by heating at 58°C for 30 minutes. The decomplemented samples were serially diluted. Ten µℓ of a borate buffered saline mixture (containing 5×10^7 trypanosomes/ml) were placed on a clean microscope slide. 10 µℓ of an appropriately diluted serum sample was then added to the slide, mixed, a cover slip gently placed on the mixture, and the slide incubated at room temperature for 10 minutes. Slides were examined microscopically (@430X) for the presence of agglutinated trypanosomes. Normal NLF sera and post 1 mcd sera samples served as controls. The highest dilution of a serum sample showing a detectable agglutination reaction was recorded as the titer or end-point of the sample.

(x) Determination of Mercaptoethanol Sensitivity of Trypanosome Agglutinating Antibody. The sera samples in (ix) were made 0.1M to 2-β-Mercaptoethanol. Agglutination tests were then repeated to determine contribution by IgG to the agglutinating antibodies. (Seed, 1971).

(xi) Test for Protective Antibodies. This test, a modification of the mouse protection test (Felton, 1928), was used to determine the presence of protective antibodies. The rechallenged sera (viii,c)

were separated into IgM and IgG fractions by column chromotography utilizing a 1.5 90 cm. column packed with Sephadex G-200 and eluted with phosphate buffered saline (pH 7.8). Immunoelectrophoresis was then carried out using the standard Millipore procedure, i.e. 3µℓ of rabbit anti-rat serum (Miles laboratories) were electrophoresed in the center well of a Millipore slide for 18 minutes, 30µℓ of an IgG fraction were placed in one of the side troughs, and 30µℓ of an IgM fraction were placed in the other side trough. The sera were allowed to diffuse at room temperature in a moist chamber for three days. The slides were then washed with saline to remove unbound protein and stained with amido blue black dye (.1% in 5% acetic acid). The stained slides revealed no contamination of IgG antibodies with IgM antibodies and vice versa. Double diffusion was carried out using the two fractions and rabbit anti-rat serum: 20µℓ of each serum were placed in separate wells and allowed to diffuse for five days. The stained diffusion discs revealed no major bands of identity. Volumes (0.25ml) of IgM fractions of rechallenge sera were mixed with an equal volume (0.25ml) containing 100,000 trypanosomes and injected ip. into six male NLW mice. The same procedure was followed using the IgG fractions of rechallenge sera. Protection was recorded as the ability of sera to postpone death compared to animals receiving control fractions of normal NLF sera (viii,a) or 60 day post berenil sera plus trypanosomes.

RESULTS

(i) Duration of Berenil Prophylaxis. Control animals (no Berenil) challenged with 100,000 trypanosomes ip. exhibited parasitemia on days two through four after challenge and died on day four. Blood smears taken from all Berenil-treated animals challenged before day 28 revealed no parasites; all animals survived (Fig. 1). Of three animals challenged on day 28, one died five days after challenge, the rest survived. Two rats of each group challenged on days 28-32 after drug exhibited parasitemia and died. All animals challenged after day 32 exhibited parasitemia and died.

(ii) Determination of Trypanosome Agglutinating Antibody Titer and its Mercaptoethanol Sensitivity. Normal NLF rat serum showed a background reciprocal agglutination titer of 2. Serum from uninfected Berenil-treated animals, irrespective of day after drug administration, also showed a reciprocal titer of 2. During the course of parasitemia followed by cure antibody titers rose markedly at days three to six after initial infection (Fig. 2). The titer peaked at a reciprocal value of 1024 on days seven through nine after infection and remained at 512 on days 10-19. Trypanosome agglutinating antibodies detected during infection, cure, and after cure were mostly mercaptoethanol sensitive and attributed to the IgM class. The majority of the trypanosome agglutinating antibodies detected after rechallenge to drug induced immunity were mercaptoethanol insensitive and attributed to the IgG class (Fig. 3).

(iii) Presence of Protective Antibodies. All mice injected with the control sera fractions (normal NLF sera 60 post Berenil sera) plus trypanosomes died on the same day as animals receiving the same number of trypanosomes in buffer. A determination of one mouse protective unit was not carried out; however, mice receiving parasites mixed with IgM fractions of rechallenged sera (harvested 9 days after rechallenge) lived 2-3 days longer than controls. Mice receiving IgG fractions of rechallenge sera (also harvested 9 days after rechallenge) plus parasites survived at least 4 days longer than controls.

DISCUSSION

We have found that trypanosome specific agglutinating antibodies are formed in inbred rats infected with a monomorphic strain of T. brucei and cured with Berenil. These agglutinating antibodies are predominantly of the IgM class. Our findings are consistent with those of others who have noted an increase in IgM levels in mice during infection with field strains of T. equiperdum (Mattern, Duret, and Pantrizel, 1966), and T. gambiense (Masseyeff and Lamy, 1966; Mattern, 1962). These results are also consistent with those of Seed et al (1969) who found specific agglutinating IgM antibodies to T. gambiense in rabbits. Since the IgM fraction of antibodies elicited by the T. brucei infection were not screened for reactivity or cross reactivity to heterologous antigen(s)(SRBC), the possibility of a non-specific response accompanying the specific response is not ruled out.

In the system used the duration of Berenil prophylaxis was approximately 28 days. This is consistent with the findings of Cunningham and Van Hoeve (1964) but contrasts with data on the rapid rate of drug clearance (within 24 hr.) reported by Bauer (1956). Hawking (1963) found that the in vitro trypanocidal concentration of Berenil for T. rhodesiense, T. vivax, and T. congolense was as little as 0.5µg/ml. A similar concentration could account for in vivo prophylaxis to T. brucei.

Since a predominantly mercaptoethanol insensitive IgG trypanosome specific antibody response is elicited when drug cured animals are re-challenged with homologous infective organisms a switch in production from IgM to IgG class antibodies occurs with no apparent interference

in this instance of cellular cooperativity. This contrasts with recent work on field strain infections of T. brucei (Longstaffe, Freeman, and Hudson, 1973; Murray et al, 1973) where ability by the infected host to make IgG antibodies to injected heterologous antigens is impaired, i.e. the IgM to IgG switch does not occur. Here however, it is possible that antigenic competition between the heterologous antigens and the high levels of host trypanosome antigens may occur (Katz and Bennaceraf, 1972).

Few reports have noted the humoral immune response during rechallenge of drug-cured immune animals to field-type trypanosome infections. Such studies could characterize serum factors accounting for the lack of long term immunity to reinfection. It may then be determined whether absence of immunity is attributable to immunosuppression (noted by the absence of an IgG anamnestic response to the occurring antigenic variants) or to antigenic changes in the trypanosome itself (characterized by an IgG memory response to one or more antigenic variants, yet with the emergence of an entirely new antigenic variant that is not affected by existing antibodies). Should the latter circumstance obtain then a search for common antigenic determinants among the variants and testing of the immunogenic properties of such material could be useful. Under the circumstances of the former condition immunization with trypanosomal antigen(s) may not be feasible.

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Figure 1. Duration of Berenil Prophylaxis

Rats were challenged at daily intervals after administration of a mcd (5mg/kg). Challenge dose = 100,000 trypanosomes, ip.

Figure 2. Trypanosome Agglutinating Antibodies and their Mercaptoethanol Sensitivity.

Reciprocal titers of trypanosome agglutinating antibodies occurring during infection and after Berenil cure are shown. Shaded area represents mercaptoethanol insensitive antibodies.

Figure 3. Trypanosome Agglutinating and their Mercaptoethanol Sensitivity. .

Reciprocal titers of trypanosome agglutinating antibodies formed after rechallenge to drug induced immunity is shown. Shaded area represents mercaptoethanol insensitive antibodies.

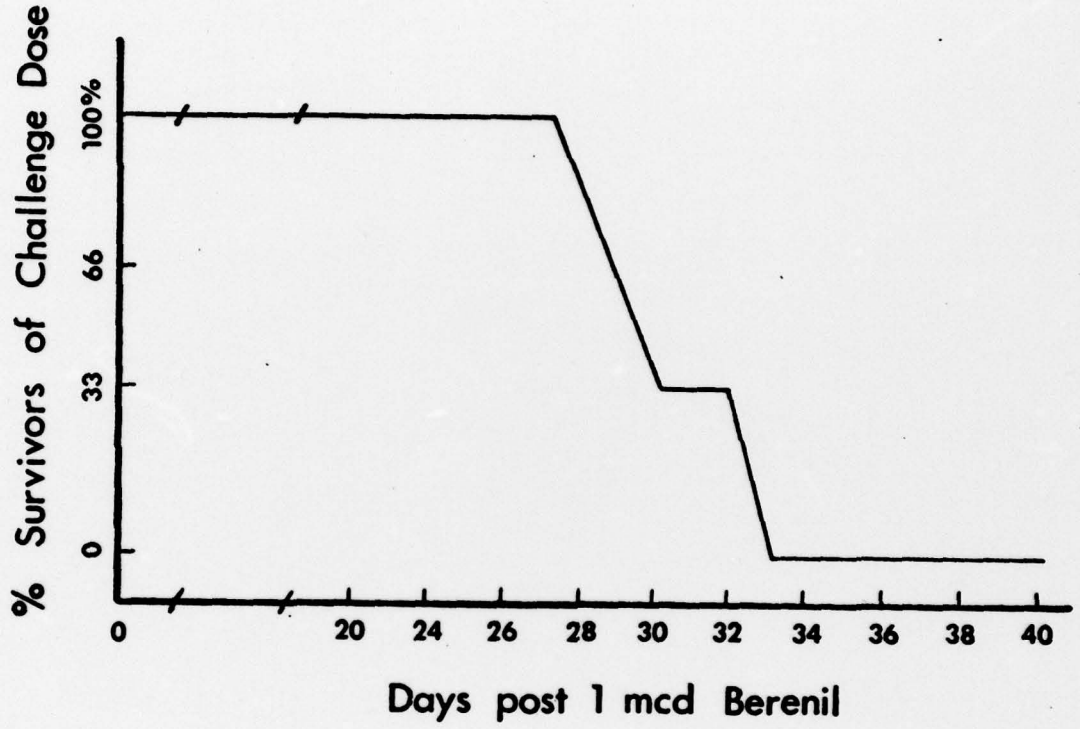
% Survivors of Challenge Dose
100%
66
33
0
0
20
24
26
28
30
32
34
36
38
40
Days post 1 mcd Berenil

FIG 1

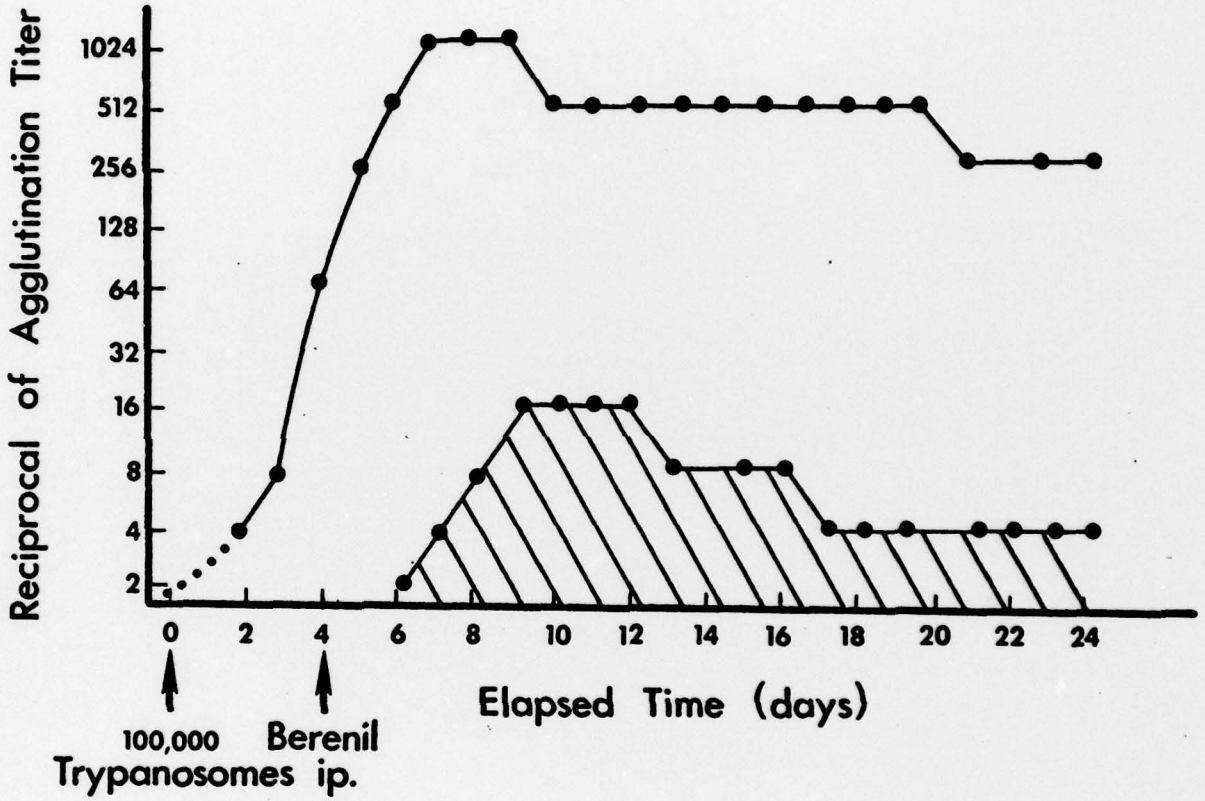
Reciprocal of Agglutination Titer
1024
512
256
128
64
32
16
8
4
2
0
2
4
6
8
10
12
14
16
18
20
22
24
Elapsed Time (days)
100,000 Trypanosomes ip.
Berenil

FIG 2

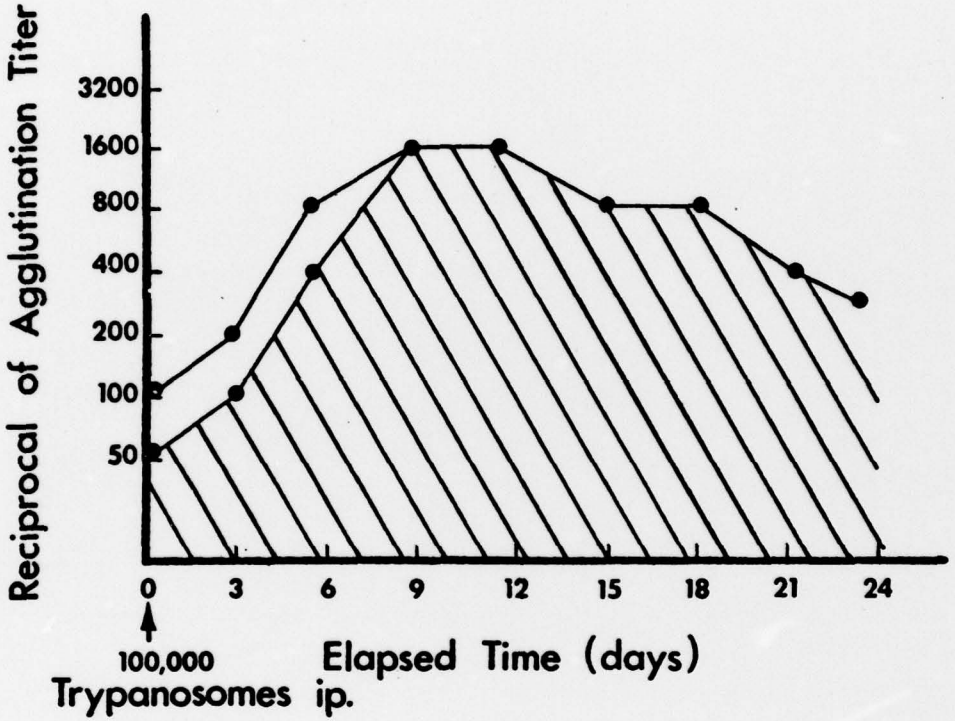
Reciprocal of Agglutination Titer
3200
1600
800
400
200
100
50
0
3
6
9
12
15
18
21
24
100,000
Trypanosomes ip.
Elapsed Time (days)

FIG 3

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